Screening fungal lectin activities from mushroom extracts with special reference on the Inocybe lectin and its specificity.

Iiro Pirhonen
University of Turku

The originality of this thesis has been checked in accordance with the University of Turku quality assurance system using the Turnitin OriginalityCheck service.

1. Abstract

Cell surface glycans are involved in many physiologically important functions such as cell differentiation and growth, cell-cell-recognition, binding and attachment, immune response and metastatic activity[1]. Under physiological conditions, a cell´s glycocalyx has a vital role for the cell to function in a normal way.
The fungus-derived lectins would offer an interesting field of research due to their affinity towards carbohydrate containing biomolecules[2]. Lectins play a role in tasks such as cell-cell attachment and cell signalling[3]. Given this, lectins could provide clinical aid due to their ability to recognize carbohydrate containing biomarkers.
The aim of this study was to screen mushroom extracts in search of lectin activities. In this study 129 mushroom samples were screened for lectin activities by using hemagglutination assay. One fungal species, Inocybe, was studied more carefully and its lectin specificity was determined. The second aim of the study was to purify the Inocybe lectin by affinity chromatography. The third aim was to analyse its specificity for several oligosaccharides and glycoproteins.

2. Methods

2.1 Preparation of the mushroom extract

50 mg of dried mushroom was homogenized into 1 ml of Phosphate Buffered Saline (PBS, 0.15 M NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4) using Dounce homogenizer. The lysate was centrifuged for 30 min, 16 000 x g at +4°C. Supernatant was used for hemagglutination assay.

2.2 Hemagglutination and hemolysis assay

Blood samples were obtained to EDTA blood collection tubes. Blood sample was centrifuged with 2000 x g for 25 minutes for removal of the buffy coat. The erythrocytes were washed with PBS for three times by centrifugation with 1000 x g, 5 min, + 4 °C. Finally, the erythrocytes were suspended to a concentration of 50 % (v/v). The same erythrocyte purification protocol was used both for screening the mushroom samples and for defining Inocybe-lectin specificity.
After the purification erythrocytes were treated with 25 mU/ml sialidase (from *Vibrio cholerae*, Roche) (25 % v/v) in PBS at 37 °C for 1 h. After treatment, the cells were washed with PBS 3 x 5 min, 1000 x g, 4 °C.
Lectin activity was tested with both untreated and sialidase treated erythrocytes by mixing 10 µl of 5 % (v/v) human erythrocytes and 10 µl of the total lysate of lectin on a cooled object glass. The hemagglutination was determined visually. Hemolysis was also checked simultaneously.

2.3 Hemagglutination inhibition assay of Inocybe extracts

Inocybe lectin carbohydrate specificity of the total lysate was also studied with glycoproteins. Several types of glycoproteins were used here: 1. Pigeon ovomucoid 1mg/ml, 2. Hen ovomucoid 1mg/ml, 3. Lactoferrin 1mg/ml, 4. Asialomucin 1 mg/ml, 5. Hen egg albumin 1 mg/ml, 6. Transferrin 1 mg/ml, 7. Bovine thyroglobulin 2 mg/ml, 8. Fetuin 1 mg/ml, 9. Bovine mucin 1 mg/ml, 10. Le^X neoglyprotein, 0.43 mg/ml, 11. Le^X-negative control protein 0.87 mg/ml 12. GalNAc (0.1 M).

2.4 Purification of Inocybe lectin

For the optimization of the affinity matrix three different carbohydrate ligand containing affinity-matrixes (D-mannose, L-fucose and lactose coupled agarose, EY Laboratories) were compared. The buffers included PBS, HEPES buffered saline (pH 7.4) and Tris buffered saline (pH 7.5). Their

properties for lectin purification by affinity chromatography was checked by elution with 200 mM of D-galactose.
50 mg of dried Inocybe was homogenized into 1 ml of either 10 mM Tris-Cl, pH 7.5, 0.15 M NaCl (TBS), 10 mM HEPES, pH 7.4, 0.15 M NaCl (HBS) or PBS. The total lysate was centrifuged for 30 min, 16 000 g at +4°C The lysate was filtered through 0.2 µm filter to remove the remaining cellular remnants.
50 µl Lactose-agarose was equilibrated in either CMF, HBS or TBS, mixed with 75 µl of the total lysate. The slurry was incubated with gentle mixing at RT. The affinity matrix was washed by centrifugation (1000 x g, 10 min, +4°C) for 4 times, incubating for 15 min at 4°C at the first time while the rest of the washing was done for 5 min each. Washing buffers were either CMF, HBS or TBS depending on the equilibration.
The lectin was eluted with 150 µl of 0.2 M galactose in buffer CMF, TBS or HBS. After incubation for 15 min at RT the matrix was separated by centrifugation and the supernatant containing the eluted lectin was stored at -20°C and the purity of the lectin was analysed with SDS-PAGE.
For determining the optimal monosaccharide for the elution, the former protocol was followed using only CMF-buffer. SDS-PAGE gels were run here as well.

2.5 Determining the carbohydrate specificity of purified Inocybe lectin

For studying the specific adhesion properties of the lectin, the lectin was diluted in CMF and mixed with oligosaccharides and glycoproteins. The inhibitors used here represented different kinds of oligosaccharides.
Hemagglutination inhibition assays were done in round bottomed microtiter plates to study lectin carbohydrate specificity. 20 µl of carbohydrate was diluted two-fold in wells and 20 µl of purified lectin (corresponding to a minimum agglutinating dose) was added. Finally, 40 µl of 5 % (v/v) erythrocytes were added. The plate was incubated on ice for 2 h. The minimum inhibiting concentrations (MIC) were recorded.

3. Results

3.1 Screening of dried mushroom samples

A total of 129 mushroom extracts were screened, of which 60 were hemagglutination positive. Seven of the extracts developed a stronger hemagglutination reaction after the sialidase treatment. Those included Leucopaxillus giganteus, Clitoycybe cibba, Lepista nuda, Cortinarius caperatus, Naucoria bohemica, Volvariella gloicephala and Lactarius volemus. In addition, some fungal extracts caused hemolysis.

Table 1. The hemagglutination of fungal samples tested with untreated and sialidase treated erythrocytes. The strength of the agglutination is marked with "+++" indicating very strong, "++" strong and "+" weak hemagglutination. HL indicates hemolysis.

Fungal sample	Untreated	Sialidase
Pleurotus dryinus	+++	+++
Pleurotus ostreatus		
Pleurotus porrigens	HL	HL
Pluteus leoninus	HL	HL

Hygrocybe punicea	+++	+++
Sarcomyxa serotina	+++	+++
Pluteus atromarginatus		
Xeromphalina campanella		
Mycena galericulata		
Mycena haematopus	+++	+++
Panellus stypticus		
Macrocystidia cucumis		
Hohenbuehelia fluxilis		
Phyllotopsis nidulans		
Gymnoporus dryophilus		
Rhodocollybia maculata		
Rhodocollybia maculata		
Hohenbuehelia atrocaerulea		
Lichenomphalia umbellifera	+++	+++
Flammulina velutipes	+++	+++
Strobilurus esculentus	+++	+++
Omphaliaster borealis		
Ampulloclitocybe clavipes sample 1	+++	+++
Ampulloclitocybe clavipes sample 2	+++	+++
Hygrophorus camarophyllus	+++	+++
Hygrophorus erubescens		
Armillaria borealis		
Hygrocybe conica		
Hygrocybe punicea	+++	+++
Cheimonophyllum candidissimum		
Hygrophorus erubescens	+++	+++
Hygrocybe psittacina sample 1		
Hygrocybe psittacina sample 2	+++	+++
Hygrocybe pratensis sample 1		
Hygrocybe pratensis sample 2		
Hygrocybe pratensis sample 3	+++	+++
Cantharellula umbonata		
Hygrophorus agathosmus		
Limacella glioderma		
Hygrophorus agathosmus		
Hygrophorus camarophyllus	+++	+++
Hypsizygus ulmarius		+++
Copronus comatus		
Psathyrella candolleana	+++	+++
Lacrymaria lacrymabunda	+++	+++
Catathelasma imperiale		
Psathyrella cernua	+++	+++
Phaleopiota aurea	+++	+++
Squamanita odorata		
Entoloma serrulatum		
Calocybe gambosa		

Cystoderma carcharias	+	+
Bovista nigrescens		
Cortinatius armillatus	+++	+++
Bolbitius titubans	+++	+++
Conocybe albipes		
Coprinellus disseminatus		
Coprinopsis atramentaria		
Crucibulum laeve		
Clitocybe odora		
Leucopaxillus giganteus		+++
Clitycybe cibba		
Entoloma conferendum		+++
Lycoperdon perlatum		
Agaricus augustus	+++	+++
Asterophora lycoperdoides		
Clitopilus prunulus	HL	HL
Rugosomyces carneus		
Echinoderma aspera		
Lepiota cristata		
Lepiota castanea		
Macrolepiota procera		
Chamaemyces fracidus		
Entoloma sinuatum	HL	+++
Lyophyllum fumosum		
Melanoleura verrucipes		
Leucocortinarius bulbiger	+++	+++
Lepista nuda		+++
Cortinarius caperatus		+++
Lyophyllum parustre	+++	+++
Rhodocybe nitellina		
Cortinarius venetus		
Cortinarius infractus		
Cortinarius violaceus		
Cortinarius trivialis		
Cortinarius triumphans		
Cortinarius rubellus	+++	+++
Cortinarius sanguineus		
Lentinellus concleatus		
Amanita rubescens		
Lactarius rufus	+++	+++
Chroogomphus rutilus		
Leratiomyces percevalii		
Galerina marginata		
Hebeloma mesophaeum	HL	HL
Naucoria bohemica		+++
Omphalina pyxidata		
Hypholoma fasciculare	HL	HL
Kuehneromyces mutabilis	+++	+++

Laccaria bicolor	+++	+++
Laccaria amethystina	+++	+++
Artomyces pyxidatus		
Panaeolus sphinetrinus	+++	+++
Pharorollybia lugubris	HL	HL
Pholiota flammans	+++	+++
Panaeolina foenisecii	+++	+++
Pholiota flammans	HL	HL
Psilocybe semilaunceata	+++	+++
Pholiota lubrica		+++
Deconica montana	+++	+++
Stropharia alcis	HL	+++
Auriscalpium vulgare	HL	HL
Albatrellus confluens	HL	HL
Stropharia hornemannii	HL	HL
Volvariella gloicephala	+	+++
Amanita fulva		
Tricholomopsis rutilans		
Tricholoma pessudatum	+++	+++
Tricholoma equestre	HL	HL
Lactarius bertillonii		
Lactarius volemus		+++
Lactarius scrobiculatus		
Leccinum vercipelle		
Amanita regalis		
Agrocybe praecox	HL	HL
Lactarius trivialis		
Amanita virosa	HL	HL
Russula foetens	+++	+++
Russula chloroides		
Russula badia		
Russula xerampelina		
Russula emetica		
Russula decolorans		

3.2 Hemagglutination inhibition assay of Inocybe extract with glycoproteins

Preliminary inhibition assays with mono- and oligosaccharides showed that the lectin was specific to galactose, N-Acetylgalactosamine and lactose. The specificity was also studied with glycoproteins.

Table 2. Minimal inhibitory concentrations (MIC) of several glycoproteins tested with Inocybe total lysate.

Glycoproteins	MIC (mg/ml)
Pigeon ovomucoid	0.031
Hen ovomucoid	-
Lactoferrin	0.063
Asialomucin	0.0002
Mucin	0.0005

Hen ovalbumin	–
Thyroglobulin (bovine) 2 mg/ml	0.016
Fetuin	0.063
LeX glycoprotein	–

The strongest inhibitory glycoproteins were mucin and asialomucin. Instead lactoferrin and fetuin had high MIC values.

3.3 Optimizing the purification protocol for Inocybe lectin

Several SDS-PAGE gels were run to discover the best conditions for the lectin purification. First, three different saccharides, mannose, lactose and fucose coupled to affinity matrix were tested to find out which of the saccharides had the strongest affinity for the Inocybe lectin. Also, the effect of optimal buffer for the lectin purification was done by equilibrating the lysate into three different buffers including CMF, Tris buffered saline (TBS) and HEPES buffered saline (HBS).

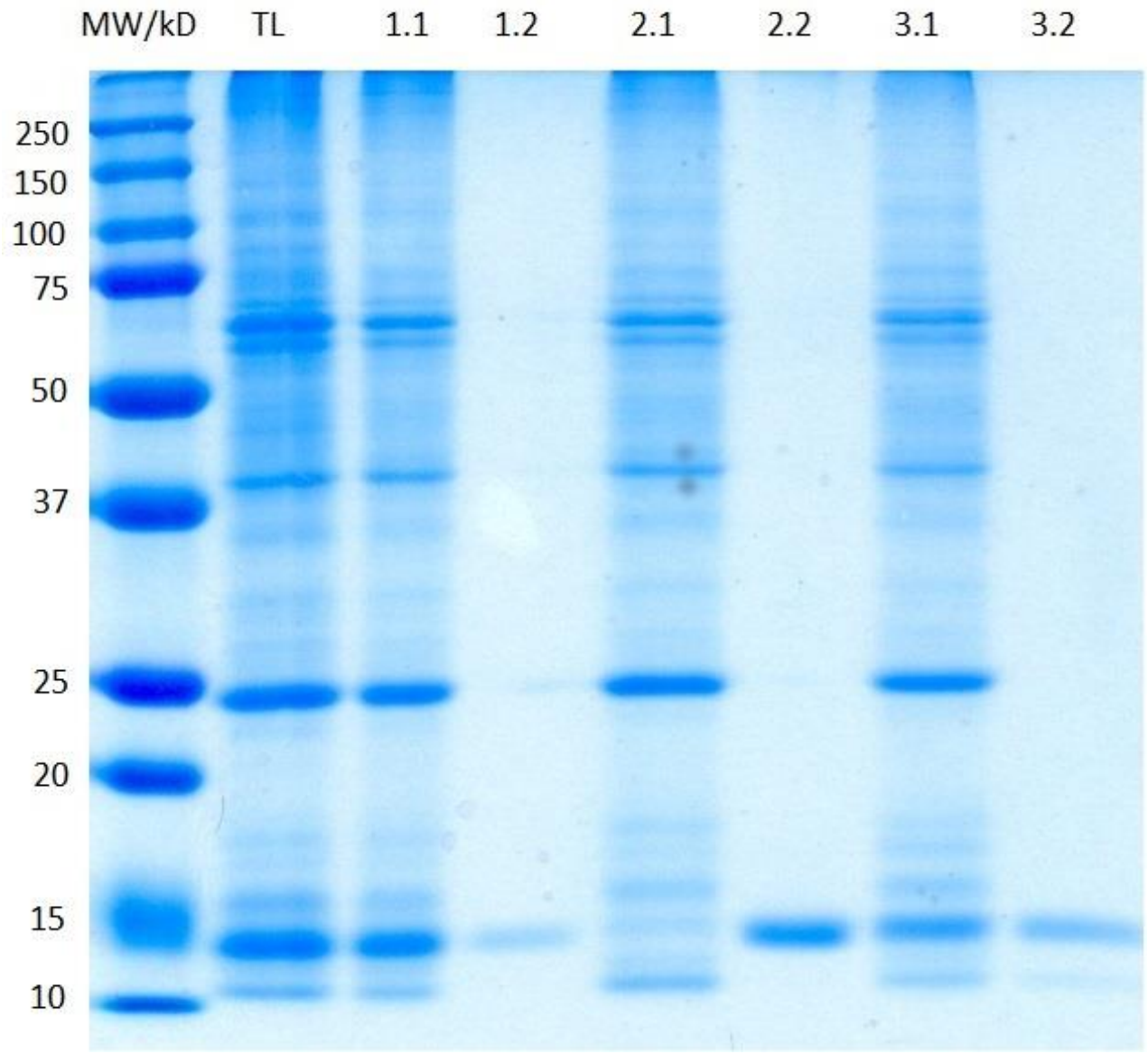

Figure 1. CMF-equilibrated affinity chromatography of Inocybe lectin. For affinity purification studies, mannose (1.1-1.2) lactose (2.1-2.2) and fucose (3.1-3.2) coupled agarose

were used. Lanes 1.1, 2.1 and 3.1 contain flow through while lanes 1.2, 2.2 and 3.2 are elution samples. Lane TL indicates the total lysate, which was common to all the samples.

SDS-PAGE analysis of the lectin purification done with different affinity matrixes (Fig. 1). It can be seen that the best affinity matrix to bind the lectin was lactose-agarose. The result was quite coherent since the tentative hemagglutination inhibition tests done with Inocybe extract showed an inhibition with lactose and galactose when tested with oligosaccharides. Since Inocybe´s strong affinity towards lactose, it was used for purifications.

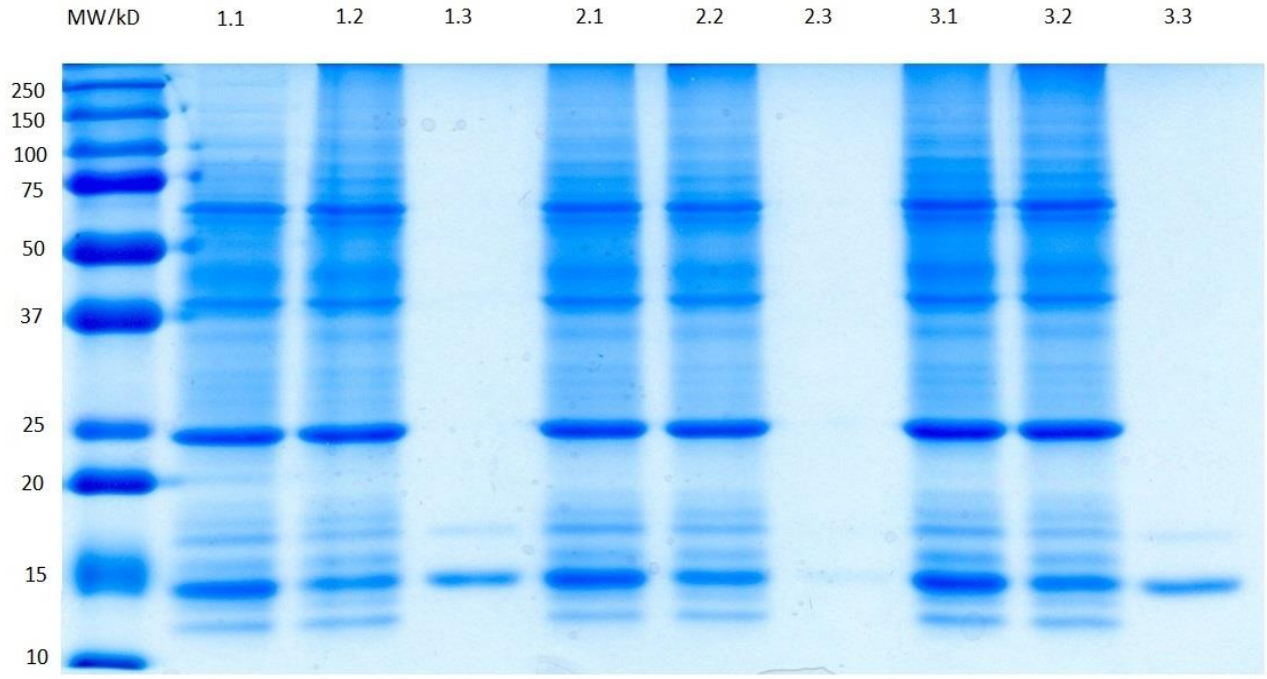

Figure 2. Purification of Inocybe lectin by affinity chromatography. 1.1 CMF-equilibrated total lysate sample. 1.2 CMF-equilibrated flow through and 1.3 elution of the same sample by using 200 mM lactose. Lanes 2.1-2.3 and 3.1-3.3 indicate Tris buffered saline (TBS) or HEPES buffered saline (HBS).

The SDS-PAGE analysis of the lectin purification with different buffers using lactose-coupled agarose (Fig. 2) show that CMF and HEPES buffers are able to purify the lectin, whereas the recovery of the lectin was significantly weaker in Tris-buffer. It can also be seen that the purified lectin protein bands were located around 15 kD, indicating the molecular weight of the lectin.

3.4 Studying the carbohydrate specificity of purified Inocybe lectin

Inocybe lectin was purified and tested with oligosaccharides (Table 3). Notable is that GalNAc had a remarkably strong inhibition of the lectin giving MIC of 0.8 mM.

Table 3. The carbohydrate specificity of the lectin studied with hemagglutination inhibition assay. (MIC, the minimal inhibitory concentration that still gave clear inhibition.)

Inhibitor	titer	MIC (mM)
D-Glc	NI[a]	–
Lac	-7	0,78
D-GlcNAc	-1	50
D-Gal	-6	1.56
D-GalNAc	-5	0.78
D-Man	-3	12.5
L-Fuc	NI	–
Maltose	NI	–
Melibiose	NI	–
Galα1–3Galα1–4Glc	-1	50
Galα1–4Galβ1–4Glc	-2	0.31
Fucα1-2Galβ1-4Glc		
GalNAcβ1–3Gal	NI	–
Raffinose	-7	0.78
Galβ1–3GlcNAcα1–3Galβ1–4Glc	-1	0.18
LeA	NI	–
LeB	NI	–
LeX	NI	–
GalNAcβ1-4(Fucα3-4)GlcNAc	NI	–

[a]NI, no inhibition

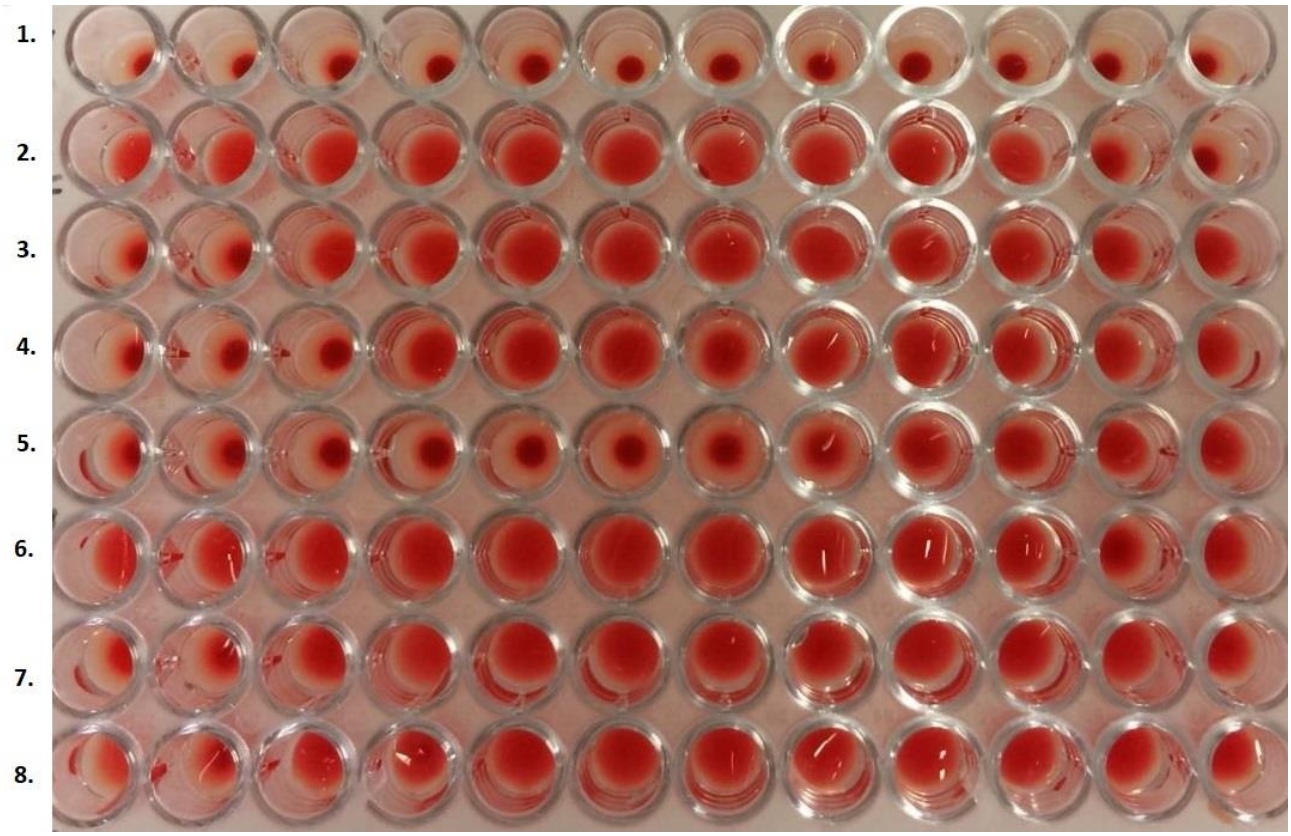

Figure 3. Hemagglutination inhibition assay of Inocybe lectin. The carbohydrate inhibitors were two-fold diluted in PBS. The oligosaccharides tested were: 1. GalNAc, 2. GalNAcβ1–3Gal, 3. Galβ1–3GlcNAcα1–3Galβ1–4Glc, 4. Galα1–4Galβ1–4Glc, 5. raffinose, 6. GalNAcβ1–3Gal, 7. Fucα1–2Galβ1–4Glc and 8. GalNAcβ1-4(Fucα3-4)GlcNAc.

4. Discussion

4.1 Screening samples

A total of 129 mushroom extracts were screened in search of hemagglutination activities. Mushrooms fulfilling certain standards have been selected for further studies, Inocybe being the most carefully studied. Even though tentative tests have been conducted, there most likely are many interesting samples left to be studied. Since the number of extracts being so high, the only possible thing to do was to narrow down the samples going for more intensive studies. The species that were selected for this study had no reports of their lectin activities. The only species described to possess lectin activity when screening through literature were Macrolepiota procera, Lactarius rufus and Laccaria amethystine. Given this, the screening project revealed a lot of new information in the field of mushroom`s lectin activities.

4.2 Studies on Inocybe

Hemagglutination assay served well on determining specific lectin properties of Inocybe. However, their lectin genes remains to be identified. This could be examined by studying the amino acid sequence of the lectin and cloning the gene from mRNA library of the Inocybe mushroom. Both total lysate and purified lectin had the same carbohydrate specificity. Weather there are more than one isoform of Inocybe lectin is still to be analysed.

4.3 General analysis and future possibilities

All in all, lectins, Inocybe among others, seem to possess interesting properties. Specific binding abilities could offer many biochemically valuable applications in the field of targeting and

haematological diagnostics. However, it is the fundamental research that creates the basis for future innovations. Also, further studies are still needed to reveal their potential applications.

5. Acknowledgements

We thank Jouni Issakainen and Jukka Vauras for collecting the fungal samples from the Biodiversity Unit of the University of Turku. Fresh samples were also collected from the field and taxonomically identified by Jukka Vauras. We also thank Sauli Haataja for supervising the thesis and providing aid both in practical field and in writing from the Institute of Biomedicine of the University of Turku.

6. References

1. Hoja-Łukowicz D., Przybyło M., Duda M., Pocheć E., Bubka M. On the trail of the glycan codes stored in cancer-related cell adhesion proteins. Biochim Biophys Acta Gen Subj. 2017 Jan;1861(1 Pt A):3237-3257. [PubMed]

2. Gabius H., André S., Jiménez-Barbero J., Romero A., Solís D. From lectin structure to functional glycomics: principles of the sugar code. Trends Biochem Sci 2011 Jun;36(6):298-313.3. Ghazarian H., Idoni B., Oppenheimer S. A glycobiology review: carbohydrates, lectins, and implications in cancer therapeutics Acta Histochem. 2011 May;113(3):236-47. [PubMed]

4. Panchak L., Antoniuk V, Purification of lectin from fruiting bodies of Lactarius rufus (Scop.: Fr.)Fr. and its carbohydrate specificity. 2007 Mar-Apr;79(2):123-8 [PubMed]

5. Santos A., Leite G., Carneiro R., Roma R., Santos V., Santos M., Pereira R., Silva R., Nagano C., Sampaio A., Rocha B., Delatorre P., Campos A., and Teixeira C. Purification and biophysical characterization of a mannose/N-acetyl-d-glucosamine-specific lectin from Machaerium acutifolium and its effect on inhibition of orofacial pain via TRPV1 receptor. Arch Biochem Biophys. 2019 Mar 30;664:149-156 [PubMed]

6. Tarbell J., Cancel L., The glycocalyx and its significance in human medicine. J Intern Med. 2016 Jul;280(1):97-113 [PubMed]

7. Kang H., Wu Q., Sun A., Liu X., Fan Y., Deng X. Cancer Cell Glycocalyx and Its Significance in Cancer Progression. Int J Mol Sci. 2018 Aug 22;19(9) [PubMed]

8. Zeng Y. Endothelial glycocalyx as a critical signalling platform integrating the extracellular haemodynamic forces and chemical signalling. J Cell Mol Med. 2017 Aug;21(8):1457-1462 [PubMed]

9. Huang M., Tota E., Verespy S., Godula K. Glycocalyx Scaffolding to Control Cell Surface Glycan Displays. Curr Protoc Chem Biol. 2018 Jun;10(2):e40. [PubMed]

10. Haataja S., Tikkanen K., Nilsson U., Magnusson G., Karlsson K., Finne J. Oligosaccharide-receptor interaction of the Gal alpha 1-4Gal binding adhesin of Streptococcus suis. Combining site architecture and characterization of two variant adhesin specificities. J Biol Chem. J 1994 Nov 4;269(44):27466-72 [PubMed]

11. Mandalasi M., Dorabawila N., Smith D., Heimburg-Molinaro J., Cummings R., Nyame A. Development and characterization of a specific IgG monoclonal antibody toward the Lewis x antigen using splenocytes of Schistosoma mansoni-infected mice. Glycobiology. 2013 Jul;23(7):877-92. [PubMed]

12. Topin J., Lelimousin M., Arnaud J., Audfray A., Pérez S., Varrot A., Imberty A. The Hidden Conformation of Lewis x, a Human Histo-Blood Group Antigen, Is a Determinant for Recognition by Pathogen Lectins. ACS Chem Biol. 2016 Jul 15;11(7): ACS Chem Biol. 2011-20. [PubMed]

13. Brazil J. Sumagin R.,Cummings R.,Louis N., and Parkos C. Targeting of Neutrophil Lewis X Blocks Transepithelial Migration and Increases Phagocytosis and Degranulation. Am J Pathol. 2016 Feb; 186(2): 297–311 [PubMed]

14. Jassam S., Maherally Z., Smith J., Ashkan K., Roncaroli F., Fillmore, H. and Pilkington G. CD15s/CD62E Interaction Mediates the Adhesion of Non-Small Cell Lung Cancer Cells on Brain Endothelial Cells: Implications for Cerebral Metastasis. Int J Mol Sci. 2017 Jul; 18(7): 1474. [PubMed]15. Hennen E, Faissner A, LewisX: a neural stem cell specific glycan? Int J Biochem Cell Biol. 2012 Jun;44(6):830-3, [PubMed]

16. Oppenheimer S., Alvarez M., Nnoli J. Carbohydrate-based experimental therapeutics for cancer, HIV/AIDS and other diseases. Acta Histochem 2008;110(1):6-13 [PubMed]